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Blood cell profile of the Indian Tree Frog *Polypedates maculatus* (GRAY, 1830), during larval development until metamorphosis
(Anura: Rhacophoridae)

Zum Blutbild von *Polypedates maculatus* (GRAY, 1830) während der Larvalentwicklung bis zur Metamorphose
(Anura: Rhacophoridae)

MADHUSMITA DAS & PRAVATI KUMARI MAHAPATRA

KURZFASSUNG

Die vorliegende Arbeit beschreibt Veränderungen in der Erythrozytenmorphologie und in der Häufigkeit verschiedener Leukozytentypen bei dem Rhacophoriden *Polypedates maculatus* (GRAY, 1830) im Verlauf der Larvalentwicklung vom Stadium der Hinterbeinknospe bis zum Abschluß der Metamorphose. Die Erythrozytenformen umfaßten sowohl normalgestaltig ovale/elliptische und runde aber auch unregelmäßige Zellen in der Form von Tränentropfen, Kommas oder mit welligem bis gezacktem Rand. Erythrozytenaggregate waren in allen untersuchten Larvalstadien zu beobachten. Von den fünf klar unterscheidbaren Leukozytentypen waren die neutrophilen Granulozyten und Lymphozyten während der frühen Larvenstadien relativ zahlreich, während das für Monozyten und eosinophile Granulozyten in den fortgeschrittenen Entwicklungsstadien zutraf. Der Anteil basophiler Granulozyten nahm während der Larvalentwicklung stetig zu. Blutplättchen traten in Klumpen auf, was die Autoren ebensowenig als Artefakt ansehen wie unregelmäßig geformte Erythrozyten und Erythrozytenaggregate. Die Untersuchung beschreibt erstmals das Blutbild der Larven von *P. maculatus*.

ABSTRACT

Changes in erythrocyte morphology and leukocyte profile were studied in the Indian Tree Frog *Polypedates maculatus* (GRAY, 1830), during larval development, from hind limb bud appearance to completion of metamorphosis. The shape of erythrocytes varied from normal oval/elliptical and round to irregular forms such as teardrop-shaped, comma-shaped, and crenulated cells which we do not think are artifacts. Aggregation of erythrocytes was observed throughout the developmental period analyzed. Out of five types of leukocytes that were clearly identified, neutrophil granulocytes and lymphocytes were comparatively numerous during the early larval stages, whereas monocytes and eosinophil granulocytes were more numerous in advanced stages of larval development. Percentage of basophil granulocytes increased gradually during the larval development. Blood platelets were found in clusters which is not classified as artifact, just as erythrocyte aggregation and irregular forms. The present study is the first to provide information on the blood cell profile of tadpoles of *P. maculatus*.

KEY WORDS

Amphibia: Anura: Rhacophoridae; *Polypedates maculatus*, leukocytes, erythrocytes, hematology, blood cell profile, India

INTRODUCTION

Metamorphosis involves structural reorganization and major physiological changes under the control of endo- and exogenous factors and often results in changes in habitat use (SANTELICS & ALVARADO 2006). In the case of anurans, the aquatic tadpoles metamorphose to terrestrial froglets. As a part of the metamorphic changes, blood cell populations are renewed (ROSENKILDE et al. 1994) and the numbers of leukocytes change (DAVIS 2009). The leukocyte components of the amphibian immune system were utilized in assessing the general health of individuals in stressful or polluted environment (CABAGNA et al. 2005; RUTHERFORD et al. 2005) and erythrocyte morphology was observed to be affected by polluted environment (BARNI et al.

2007). As anurans are considered to be environmentally sensitive animals, changes in blood cell profile could indicate the availability of contaminants in the environment (Cabagna et al. 2005; Raffel et al. 2005).

Information on the hematology of anuran larvae is largely restricted to temperate zone species. Development and ultrastructure of eosinophil, basophil and heterophil (neutrophil) granulocytes in tadpoles of *Pelophylax* snk. *esculentus* (Linnaeus, 1758) were reported (Frank 1989a, 1989b). Relative frequency of different types of leukocytes in wild-caught tadpoles of *Lithobates catesbeianus* (Shaw, 1802) were described by Davis (2009). During thyroid-induced metamorphosis of *L. catesbeianus*, changes in leukocytes were observed in the tadpoles (Jordan & Speidel 1923, 1924). Moreover, stress related changes in the number of leukocytes were found in the tadpoles of *P.* snk. *esculentus* (Barni et al. 2007), *L. catesbeianus* (Bennett & Harbottle 1968) and *Lithobates pipiens* (Schreber, 1782) (Bennett & Alspaugh 1964). Variation in erythrocyte shape and size was studied in response to the chemical stressors in the aquatic environment in *P.* snk. *esculentus* (Barni et al. 2007).

Even though the anuran fauna in India is particularly diverse in that it includes about 300 species (Anil et al. 2011; Biju et al. 2011), the blood cell profile of tadpoles has been reported only for Dubois's Tree Frog, *Polypedates teraiensis* (Dubois, 1987) by Das & Mahapatra (2012). Besides, hematological information on few adult Indian anuran species is available, namely *Duttaphrynus melanostictus* (Schneider, 1799) (Banerjee et al. 1980), *Hoplobatrachus tigerinus* (Daudin, 1802) (Mishra & Banerjee 1983) and *Polypedates maculatus* (Gray, 1830) (Mahapatra et al. 2012). To add to the knowledge of anuran larval blood, the present study characterizes ontogenetic changes in the leukocyte profile and erythrocyte morphology of laboratory-reared tadpoles of the rhacophorid Indian Tree Frog *Polypedates maculatus* (Gray, 1934).

MATERIALS AND METHODS

Fresh foam nests containing eggs of *Polypedates maculatus* were collected from a natural pond inside Utkal University campus in August 2012 at Bhubaneswar (28° 18'N, 85°50'E), Odisha, India. The hatchlings were reared in the laboratory following the procedure as described by Mohanty-Hejmadi (1977). As the holding water was conditioned tap water, its quality was not analyzed for potential contamination. The characteristics of the rearing conditions were as follows: mean temperature: 28.4 ºC, mean humidity: 89.2 %, stocking density: one tadpole reared in 100 ml of treated supplied tap water; water renewal rate: at 24 hours interval; lighting regime: 12 hours day and 12 hours night. The tadpoles were fed with boiled *Amaranthus* leaves *ad libitum*. Tadpoles from Gosner stages 26 to 46 (Gosner 1960) were selected for the present investigation. These stages are comparable to the Taylor and Kollros stages I to XXV (Taylor & Kollros 1946; McDiarmid & Altig 1999). Ten tadpoles each per developmental stage were selected for investigation. The procedure followed was described in detail by Das & Mahapatra (2012) and included anesthesia (MS-222, Tricaine Methanesulfonate, 0.0003 g/L, see Mahapatra & Mohanty-Hejmadi 1994); blood collection from mid-tail amputation (stages 26 to 44) and heart puncture (stages 45 and 46); blood smears (push slide technique); air drying; staining (Giemsa); examination under light microscope (Hund H500 Wetzlar, Germany); photodocumentation (Canon EOS 450, attached to the microscope by EF-S 18-55 1S mounting kit); microscopic blood cell differentiation (Hadji-Azimi et al. 1987; Turner 1988; Heatley & Johnson 2009) in smear regions where cells were closely associated but did not overlap (Dacie & Lewis 1984); leukocytes counts (in each smear the leukocytes of 100 grid cells were counted); erythrocytes measurements (length, width, aspect ratio and area

of cells and nuclei; ocular micrometer standardized against stage micrometer, 0.01 mm, Erma, Japan; formulae for size calculations following GRENAT et al. 2009).

Statistical analysis.– Twenty-one developmental stages (Gosner 26-46) with ten specimens per stage entered the analysis. One-way Analysis of variance was used to compare parameters across stages, with each parameter [length, width, aspect ratio and area of erythrocyte cells and nuclei; frequency (%) of lymphocytes, neutrophil, basophil and eosinophil granulocytes and monocytes] examined separately. The relationship between developmental stages and blood cell profile shown in scatter plots was subject to regression analysis. Pearson's coefficient of correlation was used to characterize the relationships between tadpole developmental stages and erythrocyte parameters. Polynomial regression of the second order was used to analyze the relationship between tadpole developmental stage and leukocyte profile. Analyses were performed using SPSS v. 11 software.

RESULTS

Blood cell morphology

The tadpoles of *Polypedates maculatus* showed some variety in erythrocyte (Red Blood Cell, RBC) morphology. Round RBCs with centrally placed nuclei (Fig. 1a), oval RBCs with centrally and eccentrically placed nuclei (Fig. 1b) and elliptical RBCs with centrally placed nuclei (Fig. 1c) were observed. Erythrocytes without nuclei (senile erythrocytes) (Fig.1d) were observed in tadpoles of stages 37 to 41. Irregular forms such as teardrop (Fig. 1e) and comma-shaped cells (Fig. 1f) were observed in tadpoles of stages 36 to 40. From stages 26 to 33, erythrocytes exhibited poikilocytosis, i.e., abnormally shaped RBCs (Fig. 1g). Dividing RBCs (Fig. 1h) were observed in tadpoles of stages 37 to 44. In climax metamorphic stages (stage 41 to 45) crenulated erythrocytes (Figs. 1i and 1j) occurred. Larger erythrocytes (Fig. 1k) were found in tadpoles of stages 36 to 42. Cells undergoing division were seen in tadpoles of stages 39 to 44 (Fig. 1l). Aggregation of erythrocytes leading to oval (Fig. 1m) or elongate structures (Fig. 1n) was observed in almost all developmental stages investigated.

The leukocytes identified included lymphocytes (large and small), monocytes, eosinophils, basophils and neutrophils. The lymphocytes were round with the nuclei occupying the entire cell leaving a narrow rim of light violet cytoplasm towards the periphery (Figs. 2a, 2b). Monocytes were round with their nuclei either centrally placed and indented (Fig. 2c) or eccentrically placed and round (Fig. 2d). Some monocytes showed eccentrically placed indented nuclei (Fig. 2e) as well. Few monocytes with irregular edges were observed (Fig. 2f). In neutrophil granulocytes, the nuclei were either trilobate (Fig. 2g) or tetralobate (Fig. 2h). Eosinophil granulocytes had bilobate nuclei, located at one end of the cells (Fig. 2i). Basophil granulocytes had large dark violet-stained granules scattered over the entire cell, including the irregular nuclei (Fig. 2j). Blood platelets were found in clusters (Fig. 2k).

Length, width and areas occupied of the RBCs and their nuclei are shown in Table 1 for larval developmental stages 26 through 46. Frequency of the of leukocytes types at larval developmental stages 26 through 46 are shown in Table 2.

Statistical analysis

Statistical comparisons of the various study parameters across 21 developmental stages showed significant variation in all parameters (Table 3, Figs. 3 and 4). Length, width and area of the erythrocytes were negatively correlated with developmental stage (Figs. 3a, 3b, 3d). Aspect ratio, i.e., length/width ratio, of the cell was positively correlated with developmental stage (Fig. 3c). In case of the nucleus, all the parame-

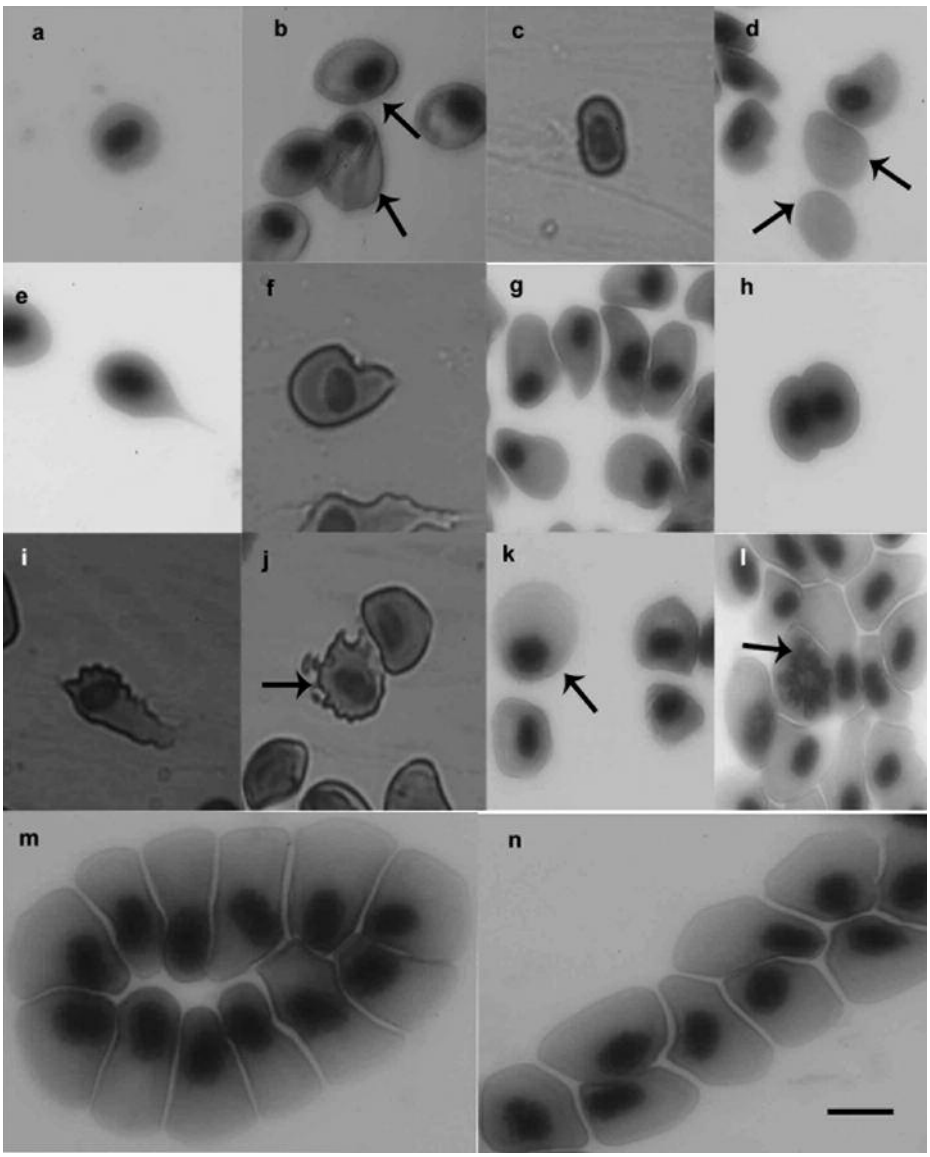

Fig. 1: Erythrocytes of the tadpoles of *Polypedates maculatus* (Gray, 1830).
a - Round cell with centrally placed nucleus, b - oval cells with centrally and eccentrically placed nuclei, c - elliptical cell with centrally placed nucleus, d - senile erythrocytes (without nucleus), e - teardrop-shaped cell, f - comma-shaped cell, g - poikilocytosis, h - dividing erythrocyte, i and j - crenulated erythrocytes, k - large erythrocytes, l - erythrocyte undergoing cell division, m and n - aggregation of erythrocytes. Length of scale bar represents 10 µm.

Abb. 1: Erythrozyten der Larven von *Polypedates maculatus* (Gray, 1830).
a - Runde Zelle mit zentralem Kern, b - ovale Zellen mit zentral und exzentrisch gelegenen Kernen, c - elliptische Zelle mit zentralem Kern, d - senile (kernlose) Erythrozyten, e - tränenförmige Zelle, f - kommaförmige Zelle, g - Poikilozytose, h - Erythrozyt in Teilung, i und j -Erythrozyten mit gezackten oder welligen Zellgrenzen, k - große Erythrozyten, l - Erythrozyt in Zellteilung, m und n - Erythrozytenaggregate. Die Balkenlänge entspricht 10 µm.

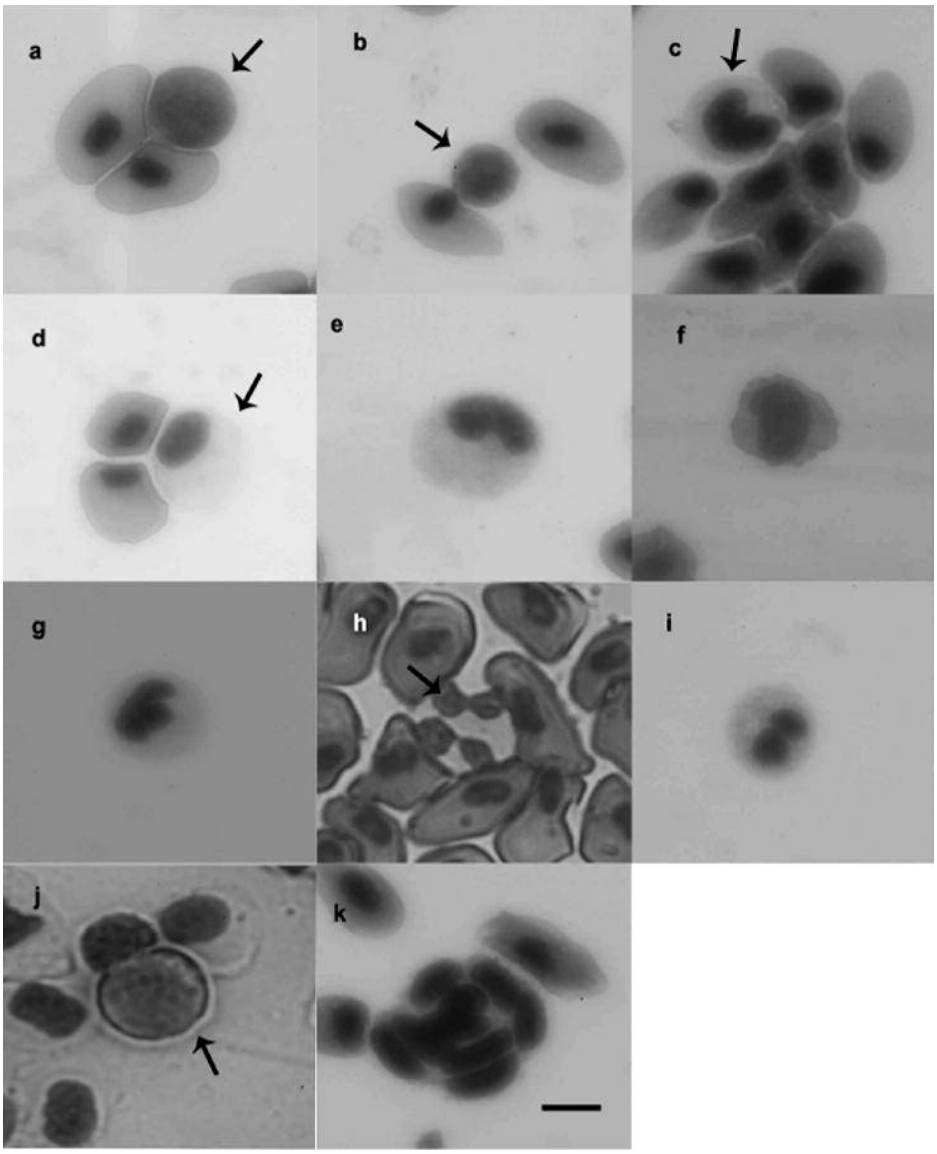

Fig. 2: Leucocytes and platelets of the tadpoles of *Polypedates maculatus* (Gray, 1830).
a - large lymphocyte, b - small lymphocyte, c - monocyte with centrally placed indented nucleus, d - monocytes with eccentrically placed round nuclei, e - monocyte with eccentrically placed indented nucleus, f - monocytes with irregular cell edges, g - neutrophil granulocyte with trilobate nucleus, h - neutrophil granulocyte with tetralobate nucleus, i - eosinophil granulocyte, j - basophil granulocyte, k - platelets in cluster. Length of scale bar represents 10 µm.

Abb. 2: Leukozyten und Blutplättchen der Larven von *Polypedates maculatus* (Gray, 1830).
a - großer Lymphozyt, b - kleiner Lymphozyt, c - Monozyt mit zentral gelegenem, gekerbten Kern, d - Monozyten mit exzentrischen, runden Kernen, e - Monozyt mit exzentrischem, gekerbten Kern, f - Monozyten mit unregelmäßigen Zellgrenzen, g - Neutrophiler Granulozyt mit dreilappigem Kern, h - neutrophiler Granulozyt mit vierlappigem Kern, i - eosinophiler Granulozyt, j - basophiler Granulozyt, k - verklumpte Blutplättchen. Die Balkenlänge entspricht 10 µm.

Table 1: Morphometry of erythrocytes in the blood of *Polypedates maculatus* (Gray, 1934) at 21 defined (Gosner 1960) larval developmental stages. Arithmetic mean values ± one standard deviation (SD) are given. The number of erythrocytes measured was 200 cells per developmental stage.

Tab. 1: Morphometrie der Erythrozyten im Blut von *Polypedates maculatus* (Gray, 1934) bei 21 definierten (Gosner 1960) larvalen Entwicklungsstadien. Angegeben sind der arithmetische Mittelwert ± eine Standardabweichung (SD). Die Anzahl vermessener Erythrozyten betrug 200 je Entwicklungsstadium.

Developmental stage		Size of erythrocyte cell				Size of erythrocyte nucleus				A' / A
Gosner (1960)	Taylor & Kollros (1946)	Length (L ± SD) [µm]	Width (W ± SD) [µm]	L/W ± SD	Area (A ± SD) [µm²]	Length' (L' ± SD) [µm]	Width' (W' ± SD) [µm]	L'/W' ± SD	Area' (A' ± SD) [µm²]	
26	I	20.54±0.52	18.25±0.78	1.12±0.05	292.883±14.76	9.99±0.73	9.69±0.51	1.03±0.05	74.81±8.92	0.25±0.02
27	II	21.68±0.84	18.18±0.56	1.19±0.04	307.013±17.27	9.09±0.81	9.09±0.81	1±0.09	63.82±10.36	0.20±0.04
28	III	19.27±0.63	16.55±0.52	1.16±0.05	248.688±11.31	9.09±0.81	8.89±0.96	1.05±0.06	61.23±12.22	0.24±0.04
29	IV	19.47±0.51	17.85±0.78	1.09±0.05	271.139±15.48	10.23±0.63	9.99±0.31	1.03±0.04	79.36±6.66	0.29±0.03
30	V	21.54±0.52	18.56±0.52	1.16±0.02	312.351±14.81	10.29±0.42	9.09±0.0	1.13±0.04	72.06±2.97	0.23±0.01
31	VI	20.16±0.56	17.25±0.63	1.16±0.98	271.453±14.05	9.98±0.98	8.63±0.98	1.15±0.31	67.03±9.00	0.24±0.03
32	VII	19.57±0.84	17.15±0.56	1.14±0.05	261.876±16.69	9.96±0.89	8.96±0.87	1.08±0.05	67.51±12.26	0.25±0.03
33	VIII	21.96±0.56	18.96±0.56	1.15±0.02	325.068±16.66	9.63±0.51	8.69±0.51	1.11±0.00	64.99±7.29	0.20±0.02
34	IX	20.35±0.48	19.25±0.63	1.05±0.01	306.15±16.95	9.09±0.47	8.60±0.69	1.05±0.06	61.62±7.04	0.20±0.18
35	X	20.56±0.52	18.5±0.52	1.10±0.00	297.907±16.93	9.45±0.51	9.09±0.0	1.04±0.05	66.41±3.64	0.22±0.01
36	XI	22.68±0.51	18.96±0.73	1.19±0.05	335.352±16.38	9.39±1.05	9.12±0.87	1.02±0.07	66.96±13.54	0.19±0.03
37	XII	21.65±0.84	19.21±0.63	1.12±0.03	325.775±20.55	9.98±0.56	8.56±0.52	1.16±0.06	66.17±6.94	0.20±0.01
38	XIII	19.56±0.52	17.86±0.63	1.09±0.04	272.473±12.27	10.12±0.31	9.69±0.51	1.05±0.07	76.06±3.74	0.27±0.01
39	XIV	20.87±0.63	16.63±0.51	1.25±0.05	271.060±12.24	9.09±0.0	9.09±0.0	1±0.0	63.58±0.0	0.23±0.01
40	XV-XVII	21.33±0.67	17.89±0.63	1.14±0.05	283.699±14.64	9.10±0.31	9.09±0.0	1.01±0.03	64.29±2.23	0.22±0.01
41	XVIII-XIX	21.65±0.51	18.89±0.51	1.16±0.04	315.334±10.06	8.98±0.31	8.36±0.67	1.07±0.06	58.09±6.08	0.18±0.01
42	XX	20.62±0.69	16.63±0.51	1.24±0.07	268.313±8.76	8.26±0.63	8.26±0.63	1±0.00	53.06±8.11	0.19±0.03
43	XXI	20.35±0.67	15.53±0.52	1.31±0.04	247.110±14.20	8.56±0.52	7.98±0.56	1.07±0.06	53.53±7.02	0.21±0.03
44	XXII	20.65±0.51	16.33±0.67	1.26±0.08	263.367±5.86	8.39±0.67	7.99±0.31	1.05±0.08	51.49±4.95	0.19±0.01
45	XXIII-XXIV	19.93±0.87	15.45±0.69	1.29±0.07	240.681±17.22	8.63±0.51	8.13±0.31	1.06±0.08	54.63±3.03	0.22±0.01
46	XXV	18.96±1.19	14.12±0.56	1.34±0.09	209.281±16.74	8.69±0.51	7.98±0.87	1.09±0.06	53.61±8.79	0.25±0.05

Table 2: Percentage of leukocytes in the blood of *Polypedates maculatus* (Gray, 1934), at 21 defined (Gosner 1960) larval developmental stages. Arithmetic mean values ± one standard deviation (SD) are given. The number of leukocytes measured was 1000 cells per developmental stage.

Tab. 2: Prozentsatz der Leukozyten im Blut von *Polypedates maculatus* (Gray, 1934) bei 21 definierten (Gosner 1960) larvalen Entwicklungsstadien. Angegeben sind der arithmetische Mittelwert ± eine Standardabweichung (SD). Die Anzahl vermessener Leukozyten betrug 1000 je Entwicklungsstadium.

Developmental stage Gosner (1960)	Taylor & Kollros (1946)	Lymphocytes Mean ± SD	Neutrophil granulocytes Mean ± SD	Monocytes Mean ± SD	Eosinophil granulocytes Mean ± SD	Basophil granulocytes Mean ± SD
26	I	69.9 ±0.96	22.1±0.68	1±0	5.8±0.01	1.2±0.10
27	II	65.3±0.89	22.6±0.68	2.1±0.12	6.4±0.11	3.6±0.10
28	III	63.2±0.99	23.9±0.85	2.1±0.11	7.3±0.12	3.5±0.12
29	IV	62.9±0.56	24.2±0.67	2.1±0.13	8±0.25	2.8±0.01
30	V	63.5±1.2	21±0.56	2.7±0.11	7.6±0.55	5.2±0.15
31	VI	61.3±1.56	19.2±1.23	4.2±0.23	9.2±0.55	6.1±0.25
32	VII	62.1±0.97	18.8±0.59	4.1±0.23	8.1±0.75	6.9±0.25
33	VIII	63.1±1.3	18±0.55	4.8±0.25	8±1.2	6.1±0.35
34	IX	63.9±1.25	15.2±0.68	7.5±0.31	6.1±1.1	7.3±0.55
35	X	66.9±1.56	15.9±0.96	6.8±0.35	5.2±0.25	5.2±0.55
36	XI	65.1±0.59	16.9±1.23	6.1±0.35	5.1±0.11	6.8±0.85
37	XII	62.3±1.54	15±0.85	6.8±0.36	8.6±0.56	7.3±0.65
38	XIII	58.3±2.3	15.9±0.55	8.9±0.45	9.9±0.85	7±0.75
39	XIV	58.8±2.1	15.9±0.85	9.1±0.11	10.6±1.2	5.6±0.11
40	XV-XVII	56.9±1.53	17.1±0.74	9±1.2	8.9±1.1	8.1±0.25
41	XVIII-XIX	57.6±1.25	18.3±0.75	6±0.36	10±1.2	8.1±0.55
42	XX	56.9±1.69	19.2±0.29	5.5±0.11	10.3±1.11	8.1±0.86
43	XXI	54.1±1.65	16.1±0.12	6.8±0.12	13.9±0.13	9.1±0.12
44	XXII	56.6±1.23	19.2±0.11	5.8±0.11	11.2±0.25	7.2±0.12
45	XXIII-XXIV	54.2±1.55	18.5±0.55	8.1±0.35	11.1±0.55	8.1±0.15
46	XXV	53.2±1.23	19.6±0.55	8.3±0.55	10.8±0.75	8.1±0.25

Table 3: Statistics characterizing the numerical relationship between erythrocyte morphological parameters as well as leukocyte type frequencies versus developmental stage in tadpoles of *Polypedates maculatus* (Gray, 1830). The data of 210 tadpoles of 21 developmental stages (Gosner 1960) was analyzed. $F_{(20,189)}$ - *F*-test value; (*p*) - probability value of the *F*-test; (*r*) - Pearson's coefficient of correlation, *p*-value - significance of the probability value of Pearson's correlation coefficient, ANOVA (R^2) - coefficient of determination, ANOVA (F) - *F*-test.

Tab. 3: Statistiken zur Kennzeichnung der numerischen Beziehung zwischen morphologischen Erythrozytenmerkmalen sowie der Häufigkeit bestimmter Leukozytentypen einerseits und dem Entwicklungsstadium andererseits bei den Larven von *Polypedates maculatus* (Gray, 1830). Die Daten von 210 Kaulquappen aus 21 Entwicklungsstadien (Gosner 1960) wurden untersucht. $F_{(20,189)}$ - Wert des *F*-Tests; (*p*) - Wahrscheinlichkeitsniveau für den Wert des *F*-Tests; (*r*) - Pearson's Korrelationskoeffizient, *p*-value - Signifikanz des Wahrscheinlichkeitswerts für den Wert von Pearson's Korrelationskoeffizienten, ANOVA (R^2) - Bestimmtheitsmaß, ANOVA (F) - *F*-Test.

Blood cell parameter measured	$F_{(20, 189)}$	(*p*)	(*r*)	*p*-value	ANOVA (R^2)	ANOVA (*F*)
Erythrocyte cell length [µm]	20.99	≤ 0.0001	-0.101	0.663		
Erythrocyte cell width [µm]	50.67	≤ 0.0001	-0.533	0.012		
Erythrocyte cell area [µm²]	47.98	≤ 0.0001	-0.409	0.065		
Erythrocyte cell aspect ratio (length/width)	50.67	≤ 0.0001	0.652	0.001		
Erythrocyte nucleus length [µm]	10.20	≤ 0.0001	-0.686	0.0005		
Erythrocyte nucleus width [µm]	9.41	≤ 0.0001	-0.680	0.0006		
Erythrocyte nucleus area [µm²]	5.957	≤ 0.0001	-0.042	0.856		
Erythrocyte nucleus aspect ratio (length/width)	10.53	≤ 0.0001	-0.718	0.0002		
Lymphocytes frequency (%)	12.93	≤ 0.0001			0.7815	67.95
Neutrophil granulocytes frequency (%)	5.17	≤ 0.0001			0.7452	55.55
Basophil granulocytes frequency (%)	8.70	≤ 0.0001			0.8097	80.84
Eosinophil granulocytes frequency (%)	18.60	≤ 0.0001			0.5778	26.00
Monocytes frequency (%)	22.12	≤ 0.0001			0.7881	70.66

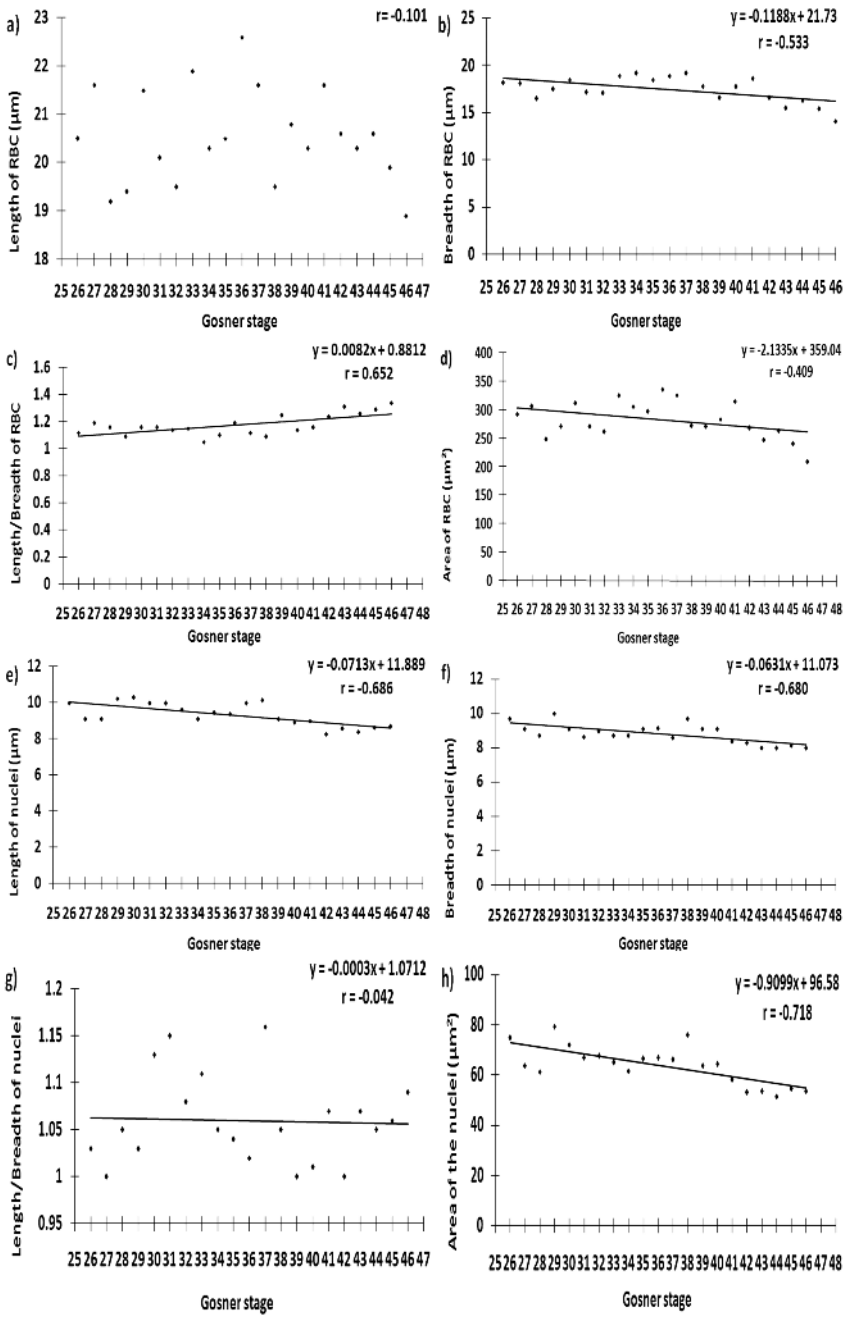

Fig. 3: Correlation between different morphometric values of erythrocytes and numeric developmental stage of tadpoles of *Polypedates maculatus* (Gray, 1830).

Abb. 3: Korrelation zwischen den untersuchten morphometrischen Erythrozytenmerkmalen und dem numerischen Entwicklungsstadium bei Larven von *Polypedates maculatus* (Gray, 1830).

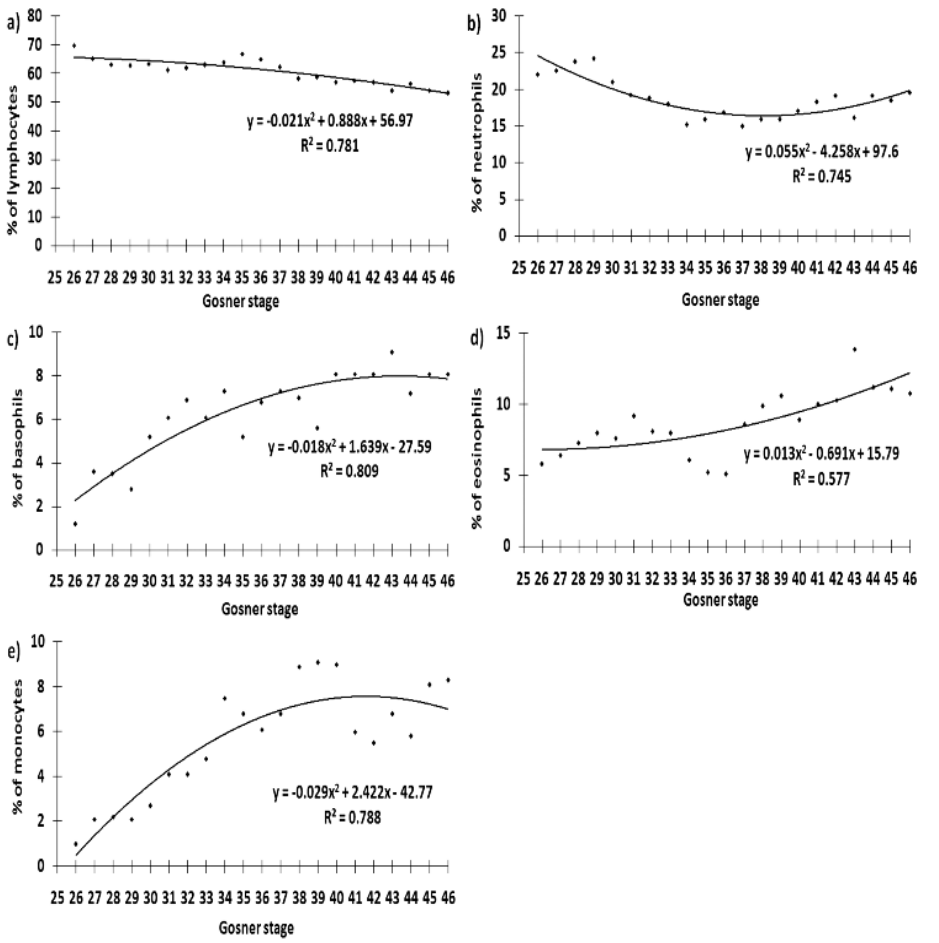

Fig. 4: Polynomial regression of the second order describing the relationship between percentage of different types of leucocytes and numerical developmental stage of tadpoles of *Polypedates maculatus* (GRAY, 1830).

Abb. 4: Polynomiale Regression zweiter Ordnung zur Beschreibung der Beziehung zwischen dem Prozentsatz verschiedener Leukozytentypen und dem numerischen Entwicklungsstadium bei Larven von *Polypedates maculatus* (GRAY, 1830).

ters showed a negative correlation that increased with the tadpole development (Figs. 3d, 3e, 3f, 3g).

The percentage of lymphocytes varied throughout the developmental period (Fig. 4a). Neutrophil granulocytes percentage varied throughout the developmental period and was highest during early stages (Fig. 4b). Frequency of basophil granulocytes increased throughout developmental period (Fig. 4c). The number of eosinophil granulocytes increased gradually, attaining maximum values at climax stages of metamorphosis (Fig. 4d). Monocyte frequency increased gradually, until it settled at a high level in tadpoles of stages 34 to 46.

DISCUSSION

Blood cell morphology

The erythrocytes were morphologically heterogeneous as they appeared in various shapes such as round, oval or elliptical. Similarly, the position of the nuclei within these cells also varied. The nuclei were either placed eccentrically (Fig. 1b) or centrally (Figs. 1a, 1c) within the cells. About 20 % of the erythrocytes showed deviations from the normal shape. Such increased variation in shape of the erythrocytes (poikilocytosis) was evident at the early larval developmental stages 26 to 33 (Fig. 1g) but was no longer observed at later stages, suggesting poikilocytosis to be a normal phenomenon during the early larval period. Some specified forms of erythrocytes such as teardrop-shaped cells (Fig. 1e) and comma-shaped cells (Fig. 1f) were observed at stages 36 to 40. Crenulated cells (Figs. 1i, 1j) characterized by protrusion of spiny processes from the external surface were seen in the tadpoles of stages 41 to 45. These cells resembled the echinocytes and acanthocytes seen in mammals (Foglia 2010). Similar crenulated cells were reported in *Lithobates pipiens* (Hollyfield 1966) and *Polypedates teraiensis* (Das & Mahapatra 2012). In red blood cells, metabolic stress produces crenulated cells; this type of transformation of shape is reversible. Small crenulated erythrocytes appear during metamorphosis, increase in number as the larval development proceeds and gradually lose their wrinkled appearance (DeGruchy 2008). In thyroxin-treated tadpoles of *Lithobates catesbeianus*, Vankin et al. (1970) observed the RBC outlines to be more irregular and crenulated with many cytoplasmic projections and correlated this phenomenon with anemic conditions and death of the tadpoles during metamorphosis. In vertebrates, abnormally shaped red blood cells (echinocytes, acanthocytes, schistocytes, teardrop cells, and comma-shaped cells) were reported to be present during anemic conditions (College of American pathologists 2012) that ectothermic animals are capable to withstand for a long period without increased mortality (Feder & Burggren 1992). Thus, the present findings suggest the studied tadpoles to have passed through critical conditions of metabolic stress where erythrocytes underwent variation in shape. Another feature of the blood smears was the aggregation of the erythrocytes (Figs. 1m, 1n) that occurred throughout the larval period.

Senile erythrocytes (without nuclei) (Fig. 1d) were observed in the tadpoles of stages 37 to 41. Presence of senile erythrocytes during metamorphosis was reported in tadpoles of *Pseudacris crucifer* (Wied-Neuwied, 1838), *Hyla versicolor* LeConte, 1825, *Lithobates clamitans* (Latreille, 1801), *Anaxyrus americanus* (Holbrook, 1836) (Speidel 1926) and *Polypedates teraiensis* (Das & Mahapatra 2012). Erythrocytes undergoing division observed in the tadpoles from stages 39 to 44 were correlated with increased erythropoietic activity during metamorphosis as reported earlier by Maniatis & Ingram (1971) in the tadpoles of *L. catesbeianus*. The comparatively large erythrocytes in stages 36 to 42 tadpoles of the present study resembled immature erythrocytes described from tadpoles of *L. catesbeianus* (Maniatis & Ingram 1971). Similar large erythrocytes were also observed in tadpoles of stages 42 to 45 of *P. teraiensis* (Das & Mahapatra 2012).

Size (length and width) and area of erythrocytes and their nuclei were negatively correlated with developmental stage, i.e., decreased with the progress in larval development (Figs. 3a, 3b, 3e, 3f) whereas the aspect ratio i.e., L/W increased. Several works reported that during anuran metamorphosis larger larval cells were replaced by smaller adult erythrocytes (Broyles 1981; Duellman & Trueb 1986). In the present study, the area of the erythrocytes decreased along with the development of the tadpoles (Figs. 3d, h). A similar decrease in the area of the erythrocyte was reported in tadpoles of *L. catesbeianus* (Hollyfield 1966; Benbassat 1970; Hasebe et al. 1999) and *P. teraiensis* (Das & Mahapatra 2012). Decrease in area of erythrocytes during metamorphosis seems to be associated with the transition from an aquatic to a terrestrial mode of life.

Leukocyte profile

The nonspecific immune system of an organism is represented by the leukocyte population in which each cell type performs a distinct function in the immune process (Jain 1986, 1993). Out of five types of leukocytes observed in the present study, lymphocytes were the most abundant cells. With the onset of metamorphosis, the percentage of lymphocytes decreased (Fig. 4a), which suggests their particular role in the early developmental stages. In tadpoles of *L. catesbeianus* and *P. teraiensis*, the number of lymphocytes also declined with the onset of metamorphosis (Davis 2009; Das & Mahapatra 2012). Neutrophil granulocytes were the second-most abundant type of cells after lymphocytes in the present study (Table 1). The percentage of neutrophils was highest in the early larval period (stages 26 to 34) and gradually decreased towards the climax metamorphic stages. Neutrophil granulocytes provide the "first line of defense" against invading pathogens, or any inciting inflammatory signals (Appelberg 2006). The highest percentage of neutrophils was observed in the early developmental stages, suggesting increased requirement of nonspecific immunity at the initial developmental phase, similarly seen in tadpoles of *L. catesbeianus* (Davis 2009) and *P. teraiensis* (Das & Mahapatra 2012). The percentage of monocytes gradually increased during development (Fig. 4e). At later developmental stages, there occurs more and more larval structure remodeling which results in the increase of cellular debris (Davis 2009). Increase in the number of monocytes during later stages of metamorphosis can be correlated with increase in cellular debris and need of phagocytes activity.

The percentage of eosinophil granulocytes gradually increased with the development of tadpoles advancing (Fig. 4d). Eosinophils are important in the immune system because of their antiallergic role, phagocytic function and antiparasitic actions (DeGruchy 2008). Also they are involved in the production of a number of chemical substances that initiate and modulate the immune and inflammatory response (Adamko et al. 2005; Rothenberg & Hogan 2006; Davis 2009). Thus, eosinophil granulocytes are suggested to modulate the process of tissue lysis during metamorphosis. Similarly, the proportion of basophil granulocytes increased in parallel with the development of the tadpoles (Fig. 4c). Such an increase was also reported in tadpoles of *P. teraiensis* (Das & Mahapatra 2012) and *L. catesbeianus* (Davis 2009), in which latter case the author suggested the trend to be related to these cells' formation and their entrance into the circulatory system rather than a direct association with metamorphosis.

The present investigation provides broad information on the blood cell profile of a continuous series of tadpole developmental stages of *Polypedates maculatus* which can be referred to as a reference in future investigations of blood morphology in anuran larval developmental stages.

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